

The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay

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A simple, automated test measuring the ferric reducing ability of plasma, the FRAP assay, is presented as a novel method for assessing "antioxidant power." Ferric to ferrous ion reduction at low pH causes a colored ferrous-tripyridyltriazine complex to form. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration. Absorbance changes are linear over a wide concentration range with antioxidant mixtures, including plasma, and with solutions containing one antioxidant in purified form. There is no apparent interaction between antioxidants. Measured stoichiometric factors of Trolox, α -tocopherol, ascorbic acid, and uric acid are all 2.0; that of bilirubin is 4.0. Activity of albumin is very low. Within- and between-run CVs are <1.0 and <3.0%, respectively, at 100–1000 $\mu\text{mol/liter}$. FRAP values of fresh plasma of healthy Chinese adults: 612–1634 $\mu\text{mol/liter}$ (mean, 1017; SD, 206; $n = 141$). The FRAP assay is inexpensive, reagents are simple to prepare, results are highly reproducible, and the procedure is straightforward and speedy. The FRAP assay offers a putative index of antioxidant, or reducing, potential of biological fluids within the technological reach of every laboratory and researcher interested in oxidative stress and its effects. © 1996 Academic Press, Inc.

Potentially harmful reactive oxygen species (ROS)² are produced as a consequence of normal aerobic metabolism (1, 2). These "free radicals" are usually removed or inactivated *in vivo* by a team of antioxidants (1–5). Individual members of the antioxidant defense team are deployed to prevent generation of ROS, to

destroy potential oxidants, and to scavenge ROS. Thus, oxidative stress-induced tissue damage is minimized. However, an absolute or relative deficiency of antioxidant defenses may lead to a situation of increased oxidative stress, and this may be associated with both the causes and consequences of a variety of disorders, including coronary heart disease and cancer (6–13).

Tests which measure the combined antioxidant effect of the nonenzymatic defenses in biological fluids may be useful in providing an index of ability to resist oxidative damage (13–19). Most tests of "total antioxidant power" used to date have measured the ability of plasma to withstand the oxidative effects of reactive species purposefully generated in the reaction mixture. Depletion of antioxidants is denoted by a change in signal, such as rate of oxygen utilization (17) or chemiluminescence (16–19). Measurement of these signals requires specialized equipment, and such tests can be time-consuming as well as technically demanding. This limits the availability of such tests and, furthermore, makes clinical evaluative studies difficult.

A biological antioxidant has been defined as "any substance that, when present at low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate" (20). This definition is clear and covers every member of the antioxidant defense team. However, unless an antioxidant prevents the generation of ROS, for example, by metal chelation or enzyme-catalyzed removal of a potential oxidant (1, 2), a redox reaction still occurs. The difference is that the oxidizing species reacts with the antioxidant instead of the "substrate," i.e., the antioxidant reduces the oxidant. In simple terms then, non-enzymatic antioxidants such as ascorbic acid can be described as reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one reactive species is reduced at the expense of the oxidation of another. In this context, antioxidant power may be referred to analogously as reducing abil-

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²Abbreviations used: ROS, reactive oxygen species; FRAP, ferric reducing ability of plasma; Fe^{III} -TPTZ, ferric-tripyridyltriazine.

ity. This being the case, a method using reductants in a redox-linked colorimetric method employing an easily reduced oxidant in stoichiometric excess could offer a simple way of assessing this ability.

The method described measures the ferric reducing ability of plasma (FRAP). At low pH, when a ferric-tripyridyltriazine (Fe^{III} -TPTZ) complex is reduced to the ferrous (Fe^{II}) form, an intense blue color with an absorption maximum at 593 nm develops (21, 22). The reaction is nonspecific, and any half-reaction which has a less-positive redox potential, under reaction conditions, than the $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$ -TPTZ half-reaction will drive Fe^{III} -TPTZ reduction. Test conditions favor reduction of the complex and, thereby, color development, provided that a reductant (antioxidant) is present. Ferrozine (23), a compound closely related to TPTZ, has been widely used, with excess ascorbic acid, to measure iron. In the FRAP assay, excess Fe^{III} is used, and the rate-limiting factor of Fe^{II} -TPTZ, and hence color, formation is the reducing ability of the sample.

The FRAP assay is described, and results are presented with particular reference to the following: reaction kinetics and dose-response relationships with solutions of ascorbic acid, uric acid, bilirubin, Trolox (a water-soluble analog of vitamin E), α -tocopherol, and albumin, with mixtures of these antioxidants and with plasma; relative activities of these reductants (referred to in this paper as antioxidants in line with current usage of the term) and possible interaction between them; within-run and between-run precision of the assay; FRAP values of fresh plasma from normal, healthy, Chinese adults.

MATERIALS AND METHODS

Reagent preparation. Reagents included 300 mmol/liter acetate buffer, pH 3.6 ($3.1 \text{ g C}_2\text{H}_3\text{NaO}_2 \cdot 3\text{H}_2\text{O}$ (Riedel-de Haen, Germany) and 16 ml $\text{C}_2\text{H}_4\text{O}_2$ (BDH Laboratory Supplies, England) per liter of buffer solution); 10 mmol/liter TPTZ (2,4,6-tripyridyl-s-triazine, Fluka Chemicals, Switzerland) in 40 mmol/liter HCl (BDH); 20 mmol/liter $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (BDH). Working FRAP reagent was prepared as required by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution.

Samples. Aqueous solutions of known Fe^{II} concentration, in the range of 100–1000 $\mu\text{mol/liter}$ ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; Riedel de Haen), were used for calibration. Fresh EDTA plasma from 141 consenting, healthy Chinese 21–74 years of age (mean 42 years) was chilled and assayed within 5 h of collection. "Aged plasma" was EDTA plasma that had been pooled and stored at -70°C for at least 2 months; aliquots were thawed as required and used as a between-run precision sample. All procedures involving human subjects complied with the Declaration of Helsinki of 1975, as revised in 1983.

Approval for this work was obtained from the Ethics Subcommittee of Hong Kong Polytechnic University.

Antioxidants. Solid L-(+)-ascorbic acid extra pure crystals (Merck, Germany), uric acid, solid (BDH), albumin, solid (bovine serum albumin, fraction V, Sigma), bilirubin calibrator solution (Sigma), and Trolox (Aldrich Chemical Co., U.S.A.) were used to prepare aqueous antioxidant solutions. DL- α -Tocopherol (Merck) was diluted in ethanol (Merck).

Reagent kits (Hoffman-LaRoche Ltd., Switzerland). Uric acid was measured by an enzymatic method, bilirubin was measured by an alkaline picrate method, and albumin was measured by a bromocresol green dye-binding method. These tests were performed on a Cobas Fara centrifugal analyzer (Roche) following the manufacturer's instructions and using commercially available calibrators and quality control samples.

Automated FRAP assay. A Cobas Fara centrifugal analyzer was used to perform the FRAP assay as follows: 300 μl freshly prepared FRAP reagent was warmed to 37°C and a reagent blank reading was taken (M1) at 593 nm; 10 μl of sample was then added, along with 30 μl H_2O ; final dilution of sample in reaction mixture was, therefore, 1/34. Absorbance (A) readings were taken after 0.5 s and every 15 s thereafter during the monitoring period. The change in absorbance ($\Delta A_{593\text{nm}}$) between the final reading selected and the M1 reading was calculated for each sample and related to $\Delta A_{593\text{nm}}$ of a Fe^{II} standard solution tested in parallel.

In this study the reaction was monitored for up to 8 min, but the 4-min readings were selected for calculation of FRAP values; reasons for this are discussed in a later section. Results presented for uric acid, albumin, and bilirubin are the measured concentrations. Trolox, α -tocopherol, Fe^{II} , and ascorbic acid concentrations given are the calculated concentrations. All solutions were used on the day of preparation; ascorbic acid solutions were used within 1 h of preparation. Bovine serum albumin concentrations were converted from grams per liter to micromoles per liter concentrations using a molecular mass of 68,000.

RESULTS

The FRAP assay gives fast, reproducible results with plasma, with single antioxidants in pure solution and with mixtures of antioxidants in aqueous solution and added to plasma. The dose-response characteristics of different antioxidants showed different activities, but the dose response of each individual antioxidant tested was linear, showing that activity is not concentration-dependent, at least over the concentration ranges tested in this study. The relative activity was 2.0; i.e., direct reaction of Fe^{II} gave a change in absorbance half that of an equivalent molar concentration, for Trolox, α -tocopherol, ascorbic acid, and uric acid. The activity

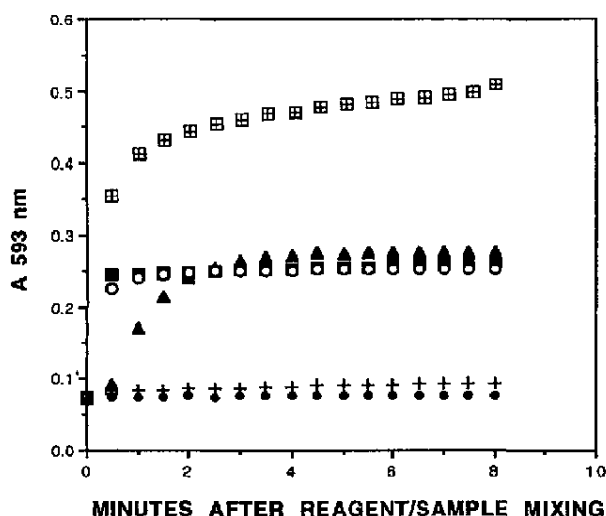


FIG. 1. FRAP reaction kinetics with individual antioxidants; rate of increase in absorbance at 593 nm for 100 $\mu\text{mol/liter}$ solutions of bilirubin (crossed squares), ascorbic acid (filled squares), uric acid (triangles), α -tocopherol (open circles), albumin (plus sign), and reagent alone (diamonds).

of bilirubin was around 4.0, while that of albumin was very low. There did not appear to be any interaction between the antioxidants tested that either enhanced or dampened antioxidant activity in this system.

Figure 1 shows the rate of increase in absorbance at 593 nm for 100 $\mu\text{mol/liter}$ solutions of bilirubin (crossed squares), ascorbic acid (filled squares), uric acid (triangles), α -tocopherol (open circles), and albumin (plus sign) compared to the monitored absorbance of reagent only (diamonds), all measured in parallel. The reaction with ascorbic acid and alpha tocopherol is very fast, reaching completion in under 1 min. The reaction with uric acid reaches an endpoint after 3 min. The reactions with the bilirubin preparation and with albumin do not reach an endpoint within the monitoring period, although the reaction of bilirubin is fast in the first few seconds and relatively slow by 2 min post-sample/reagent mixing. Glucose was tested but did not react in this system.

Because the Sigma bilirubin calibrator contains albumin (19 g/liter, 280 $\mu\text{mol/liter}$), the albumin contribution to absorbance readings of the bilirubin calibrator was explored. An aqueous solution of bovine serum albumin of 280 $\mu\text{mol/liter}$ was prepared and the FRAP reaction of this solution was monitored. The albumin-associated absorbance changes were then subtracted from those of the bilirubin calibrator run in parallel, and the albumin-corrected bilirubin absorbance changes inspected. Results are shown in Fig. 2: plus sign, bovine serum albumin (280 $\mu\text{mol/liter}$); crossed squares, albumin-containing bilirubin preparation (50 $\mu\text{mol/liter}$ bilirubin, 280 $\mu\text{mol/liter}$ albumin); open

squares, bilirubin (50 $\mu\text{mol/liter}$) corrected for the albumin-associated absorbance changes (and reagent blank-corrected). Results indicate that the continued, slow increase in absorbance seen in the reaction of the bilirubin calibrator is due to its albumin content, and that the reaction of bilirubin is complete by 4 min after sample-reagent mixing. The 0- to 4-min reaction time window, therefore, is suitable for the measurement of bilirubin's contribution to FRAP, as well as that of the other antioxidants tested.

When the test was performed with plasma but with no Fe^{III} added to the reaction mixture, no color developed. This indicates that there is no detectable free Fe^{II} in EDTA plasma and that there is no detectable agent in normal EDTA plasma that reacts directly with TPTZ to form the blue chromogen. Monitoring complete reagent, i.e., reagent containing TPTZ and Fe^{III} , but with no sample addition, showed that no color developed (Fig. 1, diamonds), indicating negligible spontaneous Fe^{III} reduction in the absence of added antioxidants.

Figure 3 shows the dose-response lines for solutions of the bilirubin preparation (crossed squares), uric acid (triangles), ascorbic acid (filled squares), α -tocopherol and Trolox (open circles), Fe^{II} (open squares), and albumin (plus sign). Each point represents the mean of four absorbance readings from four separate runs. Readings were highly reproducible, with the SD in every case ≤ 0.02 ; therefore the ± 1 SD error bars, though present in Fig. 3, do not show. The dose-response lines of α -tocopherol and Trolox were indistinguishable from each other and are both represented by a common symbol (open circle). The linear dose response obtained for

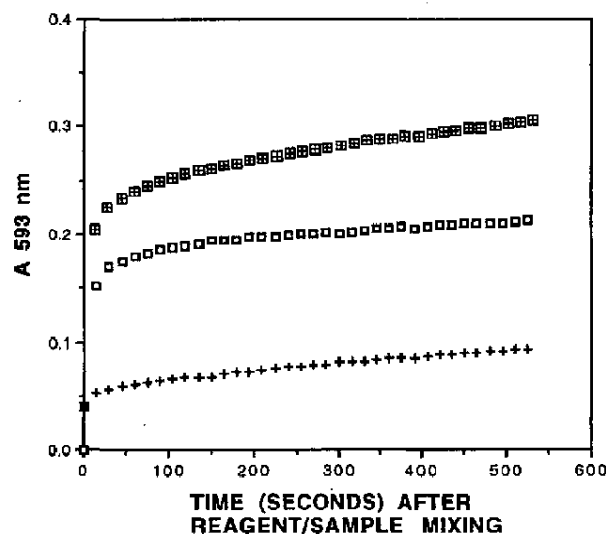


FIG. 2. Rate of increase in absorbance at 593 nm of albumin-containing bilirubin solution (crossed squares) and albumin solution (plus sign), showing the plateau of the reaction curve of bilirubin when albumin- and reagent blank-corrected (open squares).

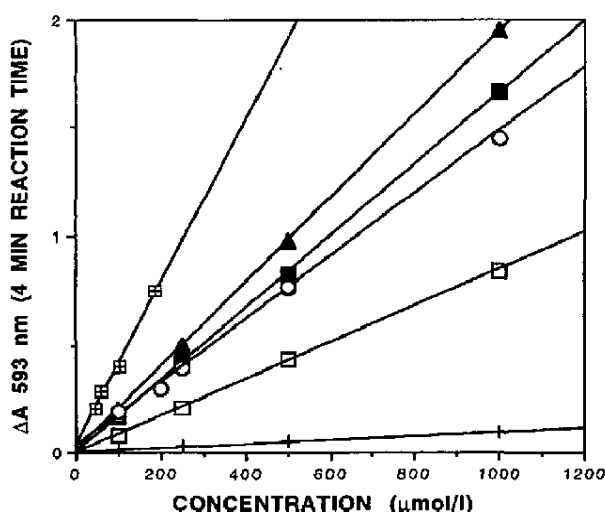


FIG. 3. Linearity of FRAP: dose-response lines for solutions of bilirubin preparation (crossed squares), uric acid (triangles), ascorbic acid (filled squares), α -tocopherol and Trolox (open circles), Fe^{II} (open squares), and albumin (plus sign).

each individual antioxidant implies that the stoichiometric factors, or antioxidant activities, of these antioxidants are not concentration-dependent in this system. However, the relative activities of the individual antioxidants in this system are not all the same. Relative to the reaction of Fe^{II} (representing a one electron exchange reaction and taken as unity), and using the 0- to 4-min reaction time window, activities of bilirubin, ascorbic acid, uric acid, α -tocopherol, Trolox, and albumin were calculated by comparing the amount of any given antioxidant with the amount of Fe^{II} required to give the same absorbance change. Calculated activities of the various antioxidant tested are given in Table 1; for α -tocopherol (and Trolox) and ascorbic acid, activities were very close to 2.0 at all concentrations tested. The activity of uric acid was slightly greater than 2.0. This is thought to be within allowable limits of experimental error, but some uric acid may be oxidized further than to allantoin, the two-electron oxidation product of uric acid.

It was of interest to investigate if the activity of albumin in the FRAP assay is truly low, or if albumin is precipitated, and so effectively removed from the reaction, by the low pH used. To explore this issue, aqueous solutions of bovine serum albumin, at concentrations of 7, 17, 34, and 68 g/liter (100, 250, 500, and 1000 $\mu\text{mol/liter}$), and five plasma samples were each diluted 1/34, the final sample dilution used in the FRAP assay, in distilled water and also in acetate buffer, pH 3.6. The albumin concentrations of the water- and buffer-diluted samples were measured, in parallel, within 15 min of dilution and also on the supernatant taken after allowing the diluted samples to stand at room tempera-

ture for 30 min followed by centrifugation for 10 min at 3000 rpm. Results are presented in Table 2. The difference due to centrifugation is negligible, and while there is a statistically significant difference between the water- and acetate buffer-diluted samples, the difference is small, at around 5 g/liter (75 $\mu\text{mol/liter}$). Thus, albumin remains in solution but has a very low reactivity in the FRAP assay, probably due to a low pH effect on protein thiol groups.

When known amounts of individual antioxidants were mixed and the FRAP value was measured, there was good recovery (91–112%). Good agreement was seen between the anticipated, by calculation, and measured FRAP values ($r = 0.990$; $P < 0.001$) after known amounts of individual antioxidants were added to plasma (Fig. 4, filled circles) and to water (Fig. 4, open circles). In addition, the FRAP dose-response relationship was the same; i.e., parallel lines were obtained, when uric acid solutions of different concentrations were tested with (Fig. 5, open triangles) and without (Fig. 5, filled triangles) the presence of 100 $\mu\text{mol/liter}$ ascorbic acid, and when different concentrations of ascorbic acid were tested with and without 200 $\mu\text{mol/liter}$ uric acid (results not shown). These results all indicate that there is no activity-changing antioxidant interaction in this test system.

Within-run coefficients of variation (CV) were $<1.0\%$ at all FRAP values tested (100, 200, and 900 $\mu\text{mol/liter}$; $n = 10$ in each case). Between-run CV was $<3.0\%$ (mean 960 $\mu\text{mol/liter}$; $n = 25$). The mean (median; SD) plasma FRAP value of 141 apparently healthy Chinese adults was 1017 (1000; 206) $\mu\text{mol/liter}$ (range, 612–1634 $\mu\text{mol/liter}$). There was a significant correlation between the FRAP values and the plasma uric acid concentrations ($r = 0.914$, $P < 0.001$; $n = 141$).

DISCUSSION

The FRAP assay is quick and simple to perform, and the reaction is reproducible and linearly related to the

TABLE 1
Relative Activity of Individual Plasma Antioxidants and Their Estimated Contributions to Total FRAP Value

Plasma antioxidant	Relative activity (measured range)	Expected ^a fasting plasma concentration, $\mu\text{mol/liter}$	Estimated % contribution to total FRAP
Ascorbic acid	2.0 (1.9–2.1)	30–100	15
α -Tocopherol	2.0 (1.7–2.1)	15–40	5
Uric acid	2.0 (2.0–2.4)	150–450	60
Bilirubin ^b	4.0 (4.2–4.6)	<20	5
Protein	0.10 (0.1–0.15)	800–1100	10
Others	—	—	5

^a From Refs. (3, 29, 35, 36).

^b Based on albumin-corrected readings.

TABLE 2
Comparison of Albumin Concentrations in Samples Prediluted in Water
with Samples Prediluted in Acetate Buffer, pH 3.6

Sample	Measured albumin concentration, g/liter (dilution corrected)			
	Predilution in water; no centrifugation	Predilution in water; postcentrifugation supernatant tested	Predilution in acetate buffer; no centrifugation	Predilution in acetate buffer; postcentrifugation supernatant tested
Bovine serum albumin, 7 g/liter	7	6	2	1
Bovine serum albumin, 17 g/liter	15	16	10	10
Bovine serum albumin, 34 g/liter	32	33	24	28
Bovine serum albumin, 68 g/liter	64	66	61	60
Plasma 1	48	48	43	44
Plasma 2	53	55	49	50
Plasma 3	52	53	48	49
Plasma 4	49	51	41	42
Plasma 5	45	46	41	43
Overall mean (SD)	41 (19)	42 (19)	35 ^a (20)	36 ^a (20)

^a Significantly different from corresponding samples diluted in water; average difference 5.2 g/liter $P < 0.01$ (test used: paired Wilcoxon rank sum test).

molar concentration of the antioxidant(s) present. There is no activity-changing interaction between antioxidants in this system nor, as has been reported (24), are stoichiometric factors concentration-dependent. Reaction mixtures containing plasma show no decrease in absorbance up to at least 30 min post-reagent/sample mixing (results not shown), indicating that there is no process or agent causing inhibition of Fe^{III} reduction or causing Fe^{II} reoxidation. It should be noted that, while ceruloplasmin in plasma has ferroxidase activity, it is unlikely to be active in FRAP due to the low pH

and high chloride concentration of the reaction mixture (25). In addition, the small, in absolute terms, physiological variations in plasma iron ($<10 \mu\text{mol/liter}$), which may occur within and between individuals, are unlikely to affect results. While the presence of large amounts of a strong iron chelator in plasma could theoretically affect results, it should be noted there is an excess of free Fe^{III} in the FRAP reagent. Free iron has a relatively low redox potential (26), and is unlikely to take part in the indicator redox reaction provided the more oxidizing Fe^{III} -TPTZ is present. In addition, it is

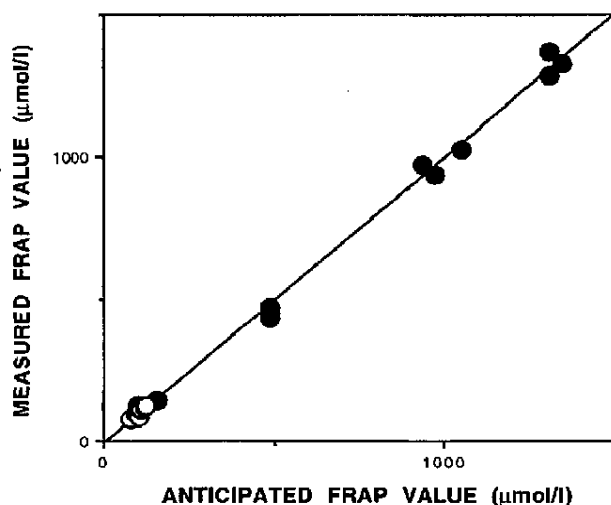


FIG. 4. Relationship between anticipated (calculated) FRAP values and measured FRAP values when known amounts of pure antioxidants were added to plasma (filled circles) and water (open circles); $r = 0.99$, $P < 0.001$.

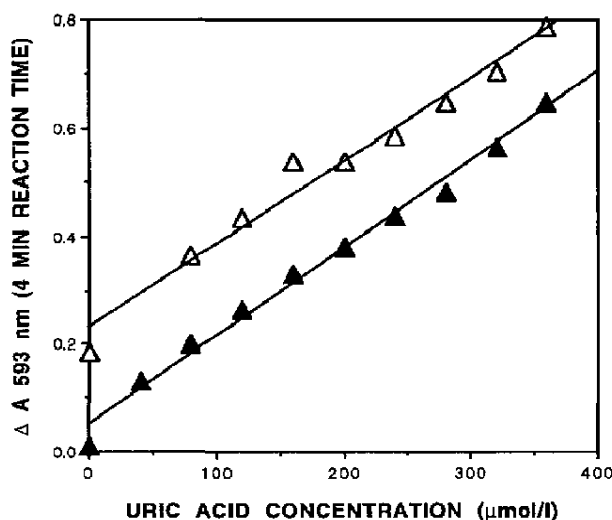


FIG. 5. Study of interaction between uric acid and ascorbic acid in the FRAP assay: the FRAP dose-response relationship of uric acid in water (filled triangles) and in an aqueous $100 \mu\text{mol/liter}$ ascorbic acid solution (open triangles).

thought unlikely that the situation would arise whereby sufficient Fe^{III} (or Fe^{II}) could be stripped from TPTZ by a chelator present in the biological sample being tested. However, if the test was performed in the presence of large amounts of an iron chelator, then increasing the amount of Fe^{III} in the reagent would probably prevent chelation interference.

In the FRAP assay, the 0- to 4 min reaction time window was used, as this encompasses the uric acid-, α -tocopherol-, ascorbic acid-, and bilirubin-related absorbance changes. Albumin reacts very slowly and its activity is low. This may be advantageous, in that FRAP values can be measured without a blanket of protein-associated absorbance changes muffling smaller, and perhaps more important, changes due to variations in other antioxidants in the sample. There is a relatively large amount of albumin in plasma (around 700 $\mu\text{mol/liter}$) and albumin may act as a sacrificial antioxidant *in vivo* (1-4). However, its role in antioxidant defense is unclear, and oxidative change in proteins may be a specific marker of oxidative damage (1-3, 8, 27) rather than a sign of antioxidant defense.

Results of this study show that FRAP values of apparently healthy Chinese adults are around 1 mmol/liter (range 0.6-1.6 mmol/liter) and correlate directly and significantly with plasma uric acid concentrations. This correlation has been reported by others (18, 28) and uric acid may have an important role in antioxidant defense (29-31) and participate in recycling of antioxidants (31-34). Using measured plasma uric acid concentrations (not shown) and a relative activity of 2.0, uric acid is estimated to contribute around 60% to the FRAP value of fresh plasma. Using the measured or expected plasma concentrations and relative activities of the other antioxidants in plasma (35, 36), the estimated relative contributions to the FRAP value of fresh plasma are 15, 5, 10, and 5% for ascorbic acid, α -tocopherol, protein, and bilirubin, respectively. These total and relative figures are similar to those of previous studies employing other methods of measuring antioxidant power (14-19, 37, 38).

The physiological importance of the apparently major contribution of uric acid to the antioxidant power of plasma is unclear and deserves further study. Also deserving of further study is the role of bilirubin as an antioxidant (39, 40). This endogenous compound, normally present in plasma at <20 $\mu\text{mol/liter}$, has an estimated antioxidant activity of 4.0 in the FRAP assay system, double that of ascorbic acid, uric acid, and α -tocopherol. While increased amounts of bilirubin in plasma are associated with disease, low concentrations have been linked to increased risk of coronary heart disease (41, 42) and the concentration of bilirubin in some areas of the body is normally and continuously high. Bilirubin may, therefore, have an important role

in protecting the liver and gut from oxidative damage and may act as a reserve or backup system, augmenting defenses when other antioxidants are lacking or depleted by oxidative stress.

As in other tests of oxidative stress and antioxidant defense, FRAP reaction conditions are far from physiological, and it must be noted that *in vitro* testing of plasma may not reflect *in vivo* hierarchies or activities (20, 26). The clinical utility of measuring antioxidant, or reducing, power in biological fluids remains to be established. Nevertheless, the FRAP assay appears to be an attractive and potentially useful test. Reagents are inexpensive, the procedure is speedy and straightforward, results are highly reproducible over a wide concentration range, and the equipment required is of a type commonly found in biochemical laboratories. Clinical studies, comparing different population groups, measuring changes in FRAP values associated with particular pathological states and monitoring FRAP values during various treatment strategies, are now needed. These are being planned, and will be supported by the data on the substantial number of normal, apparently healthy Chinese adults presented here. In addition, because the test lends itself to the measurement of reducing power in fluids other than plasma, extracts of drugs, plants, herbs, and other dietary factors can now also be studied with relative ease. To conclude, therefore, the FRAP assay offers a putative index of antioxidant defense of potential use to and within the technological reach of every laboratory and researcher interested in oxidative stress and its effects.

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